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PRINCIPAL INVESTIGATOR: Andrea R. Daniel

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, MN 55455

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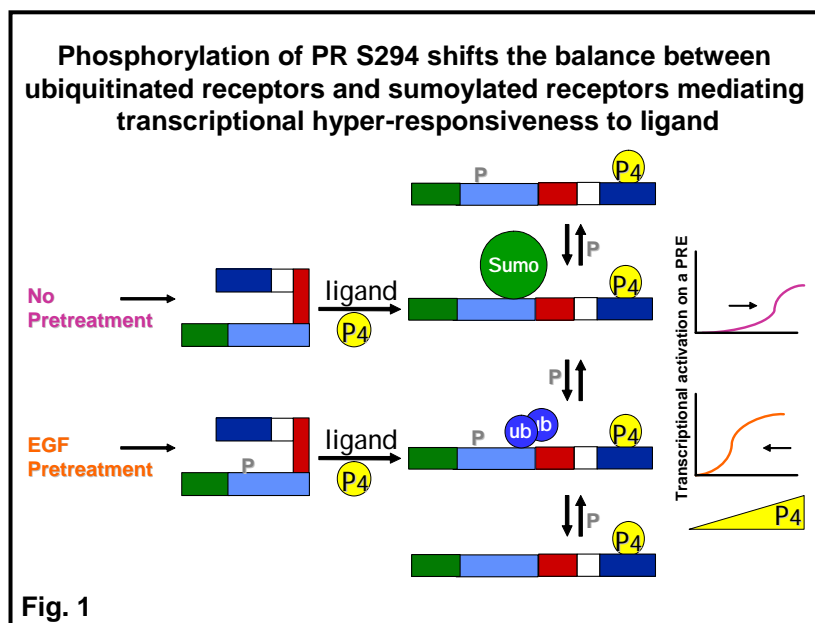
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| 14. ABSTRACT: Progesterone Receptors (PR) play important roles in both normal breast development and in the development and progression of breast cancers. PR action contributes to cell growth and survival in the presence of progestins and/or growth factors. response to ligand-binding, several kinases regulate PR via direct phosphorylation at multiple serine residues, including Ser294. The growth factor inputs can create receptors with increased transcription in response to lower concentrations of progestins, or receptors hypersensitive to ligand. Post-translational modifications such as ubiquitination and SUMOylation may be mediating these effects. goal of these studies is to elucidate the functional consequences of the post-translational modifications, specifically, phosphorylationubiquitination and SUMOylation on PR, and to determine the temporal and functional relationship between these modifications. To accomplish this goal mutants of various phosphorylation sites, conjugation sites and consensus sequences have been generated to create receptors deficient in one or more of these modifications. Our preliminary data suggests that S294A PR-B has increased sumoylation relative to wt PR-B, whereas S294A PR-B is unable to be ubiquitinated. Phosphorylation may be differentially regulating these modifications. Transcription reporter assays with S294 phosphomimic mutant PR-B display heightened transcription and the mutant deficient in sumoylation indicate SUMO is a negative regulator of PR transcriptional activity. Phosphorylation of PR by growth factor pathways differentially regulates ubiquitination and SUMOylation of the receptor mediating changes in hormone responsiveness of breast cancer. | | | | | |
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Introduction

Progesterone Receptors (PR) play important roles in both normal breast development and in the development and progression of breast cancers. Recently, the Women's Health Initiative trial (1) and the Million Women Study (2) have emphasized the role of progestins and PR in breast cancer progression with the findings that progestins added to estrogens in hormone replacement therapy (HRT) significantly increased the incidence of breast cancer in postmenopausal women. PR are ligand-activated transcription factors that contribute to cell growth in the presence of both progestins and peptide growth factors. Post-translational modifications of the receptor triggered by ligand binding or growth factor pathway activation are regulatory inputs of PR function. Several kinases regulate PR via direct phosphorylation of multiple serine residues, including S294 (3) and S400 (4). These growth factor inputs can create receptors with increased transcription in response to lower concentrations of progestins, or receptors hypersensitive to ligand. MAPK phosphorylation of PR S294 targets the receptor for ubiquitination, resulting in receptors with increased transcriptional activity and decreased stability. In addition, our preliminary data suggests that phosphorylation of PR negatively regulates its ability to be SUMOylated. SUMOylation of the receptor decreases its transcriptional activity. We hypothesize that ubiquitination of PR is the cause of the increased transcriptional activation and decreased stability; whereas SUMOylation opposes these effects by mediating a decrease in transcriptional activity and an increase in stability. Phosphorylation of PR by growth factor pathways differentially regulates ubiquitination



and SUMOylation of the receptor mediating changes in hormone responsiveness of breast cancer cells (Fig. 1).

Body

Specific Aim 1) Investigate the functional importance of the two degron consensus sites in the PR N-terminus and the lysine-rich regions, potential conjugation sites for ubiquitin. Using two-step PCR we have generated hPR1 cDNA containing mutations at the first and ninth amino acid positions of the degron consensus sites of PR-B, PR

R293A/M301A and PR R401A/N410A. We have also created a mutant PR-B containing both degron mutations to control for any compensation amongst the degrons. The double lysine sites at the hinge region and C-terminus of the hPR1 cDNA have also been mutated, PR-K641A/K642A and PR-K932A/K933A. The mutants have been sequenced for confirmation and are currently housed in the pSG5 mammalian expression vector. Transient transfection studies have shown that these PR bind ligand and shift on a polyacrylamide gel due to phosphorylation (Fig. 2). All of these mutants are functional transcription factors as they are able to activate transcription in a PRE-luciferase reporter assay (Fig. 3). These data indicate that the mutants are folded properly.

Fig. 2. PR degron and lysine mutants are phosphorylated in the presence of ligand. **A.** Cos cells were transfected with wt PR or degron mutant PR, starved for one day and treated with or without R5020 (10nM) for 1 hr. Western blots were probed for PR. **B.** Cos cells were transfected with wt PR or lysine mutant PR, starved and treated with or without R5020 (10nM) for 1 hr. Western blots were probed for PR.

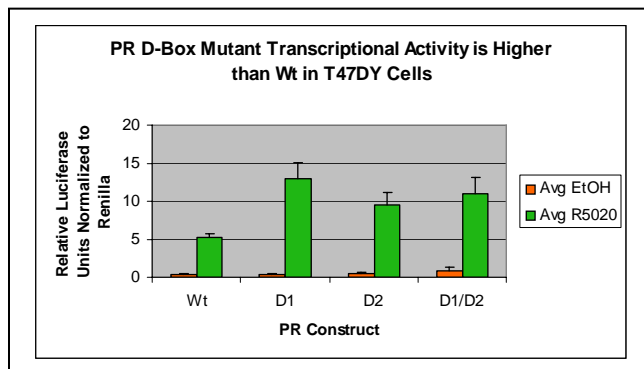
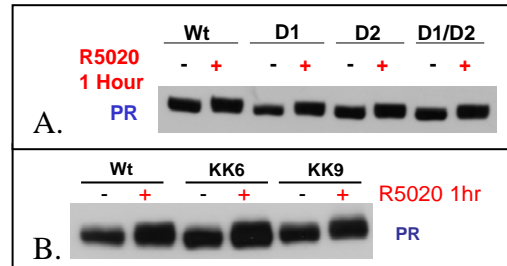


Fig. 3. PR Degron mutants are functional transcription factors. T47DY cells were cotransfected with a PR construct, a PRE-luciferase plasmid and a renilla plasmid, for transfection control. The cells were starved and treated with or without R5020 (10nM) for 24 hrs.

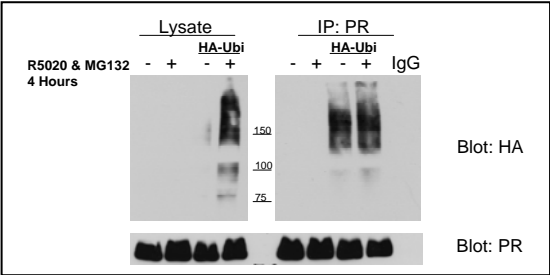
The double degron mutant has higher transcriptional activity in the presence of ligand compared to

wt in the PRE-luciferase reporter assay; there are a few potential explanations for this. One, we may have separated increased transcription from decreased stability. A recent study looking at inhibition of ubiquitination and degradation of ER shows increased transcription, conflicting with previously published data (5). Two, the transient transfection studies with overexpressed protein may not reveal the true phenotype of this mutant, as is the case with the S294A mutant, which behaves more like wt in transient PRE-luciferase assays in some cell lines. In transient assays with overexpressed PR protein the SUMO may be limiting and thus unable to exert its effects as a negative regulator of PR S294A transcription. The studies performed in the stable cell lines currently in selection will answer this question. Three, the mutant is not degraded due to defective ubiquitination and therefore increased numbers of PR are causing an increased response compared to wt. This can be determined with transient transfection luciferase assays using PR DNA concentration curves and with stable cell lines; if this is the case then this mutant will be useful for looking at regulation of ubiquitination and SUMOylation by phosphorylation. If this mutant can be phosphorylated but not ubiquitinated we can perform SUMO assays and directly determine if SUMOylation and ubiquitination are mutually exclusive due entirely to phosphorylation status. Four, the

degron mutants are not ubiquitin deficient and in this case determining why they have higher transcriptional activation may prove interesting.

Assessing the ubiquitination of these mutants is currently in progress. Ubiquitinated proteins have proven difficult to visualize due to their transient nature, their reversibility and the potentially small population of modified species at any given moment. In the Lange laboratory we are able to detect ubiquitinated PR on western blot with the inclusion of technical modifications to our regular protocols. A 30 minute pretreatment with proteasome inhibitors such as MG132 allows us to trap ubiquitinated proteins in the cell. N-ethylmaleimide (NEM) is added to our lysis buffer when harvesting the cells; this

Fig. 4. Wt PR is ubiquitinated. Cos cells were transiently transfected with wt PR and HA-tagged ubiquitin, starved for one day in serum free media and treated with or without R5020 (10nM), a synthetic progesterone, and MG132 (10uM), a proteasome inhibitor, for four hours. Cells were harvested in lysis buffer containing 5M N-ethylmaleimide (NEM) and PR was immunoprecipitated and western blots were probed with antibodies against PR and HA.



is an isopeptidase inhibitor that prevents deubiquitination. Transfecting the cells with HA-tagged ubiquitin improves our ability to detect it using antibodies. Finally, we immunoprecipitate PR and western blots are probed for both PR and HA to ensure that the bands detected are ubiquitinated PR (Fig. 4). Isotype control IgG and bead controls are used in every immunoprecipitation experiment.

Specific Aim 2) Examine the role of ubiquitination in altered PR transcriptional activity and PR protein stability relative to changes in the phosphorylation status of serine 294. Determine the proteins interacting with PR to facilitate the attachment of ubiquitin.

Activation of the MAPK pathway phosphorylates PR on S294; this localizes PR to the nucleus, targets if for ubiquitination and increases its transcriptional activation (6, 7). Ubiquitinated liganded receptors ultimately degrade rapidly. PRE-luciferase assays demonstrate that pretreatment with EGF shifts the progesterone dose response curve to the left, sensitizing the receptor to lower concentrations of R5020 (3). The S294A mutant is transcriptionally inactive and does not respond to the addition of EGF. To further investigate the hypersensitivity response we have created the opposite mutation at the S294 site. This site in PR-B was altered by two-step PCR to an aspartic acid residue, a negatively charged amino acid, to mimic phosphorylation. Transient transfection experiments, performed with and without the protein synthesis inhibitor cyclohexamide, indicate this is

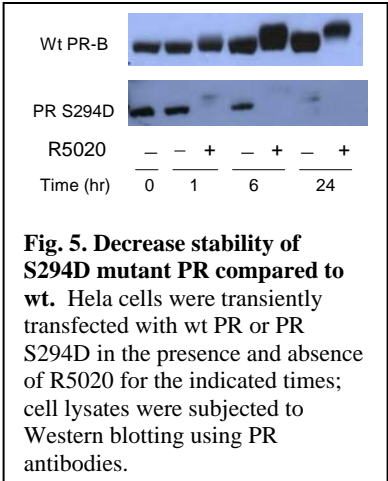


Fig. 5. Decrease stability of S294D mutant PR compared to wt. Hela cells were transiently transfected with wt PR or PR S294D in the presence and absence of R5020 for the indicated times; cell lysates were subjected to Western blotting using PR antibodies.

a less stable receptor than wt (Fig. 5) with heightened transcriptional activation (Fig. 6). T47DY cell lines stably expressing the PR-S294D mutant have recently been generated.

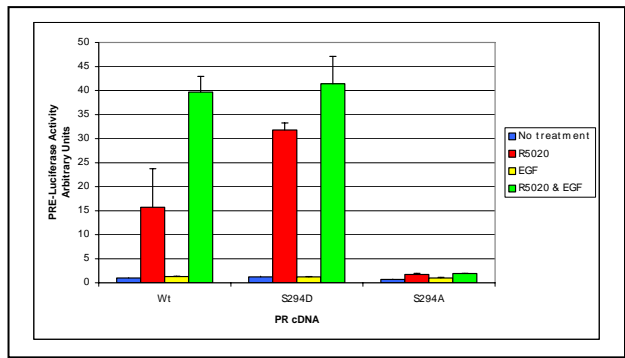


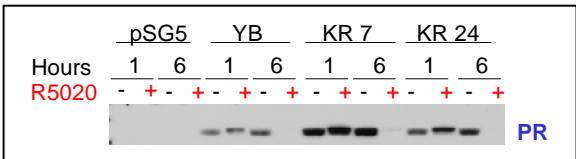
Fig. 6. Transcriptional activity of S294D is heightened compared to wt and S294A. Luciferase Assays were performed in HeLa cells cotransfected with either wt PR-B, S294D PR-B or S294A PR-B, PRE-luciferase reporter constructs and a Renilla control plasmid. Cells were treated for 24hrs with or without R5020 (10nM) and EGF (30ng/ml).

Studies investigating the effects of the phospho-regulation of PR-B SUMOylation on PR-B transcriptional activity and stability.

Preliminary data in our lab suggests that phosphorylation of Ser294 regulates another post-translational modification of PR-B, SUMOylation, in addition to ubiquitination. SUMOylation may have effects on PR-B transcriptional activity and stability that oppose those of ubiquitination. Investigating the regulation of phosphorylation and SUMOylation will give us further insight into the regulation of ubiquitination and into the mechanism of PR-B transcriptional hypersensitivity in response to growth factor inputs.

SUMO mutant PR has heightened transcriptional activity. We have created a SUMO mutant PR-B, PR K388R, housed in the mammalian expression vector pSG5 using two step PCR and confirmed it with sequencing. T47D-Y cells were then used to make cell lines stably expressing the SUMO mutant. Transfection of a plasmid containing a selectable marker gene for G418 resistance in addition to pSG5-PR K388R enabled us to make multiple cell lines expressing the mutant for comparison to the wt PR-B expressing cells, T47D-YB. Western blot analysis confirms stable expression and the PR K388R band shifting with ligand present indicates phosphorylation (Fig. 7).

Fig. 7. T47DY breast cancer cells stably expressing PR-K388R mutant. Western blot showing T47DY cell lines stably expressing vector control (pSG5), wt PR-B (YB) or PR-K388R (KR7 and KR24) treated with or without R5020 for 1 or 6 hours.



The SUMO deficient PR-K388R mutant is a functional transcription factor able to activate transcription in a PRE-luciferase reporter assay. The SUMO-mutant PR-B displays heightened transcriptional activity in response to progestins compared to wt PR-B (Fig.8), confirming previous reports (8). These data suggest that SUMO represses PR transcription on a PRE.

Fig. 8. SUMO-mutant PR display heightened transcriptional activation on a PRE. The T47D-Y cell lines stably expressing vector control, wt PR-B or K388R mutant PR-B were transfected with a PRE-luciferase plasmid and a plasmid constitutively expressing renilla luciferase for transfection control. Cells were starved for one day and treated with or without R5020 for 24 hours. Relative luciferase unit were measured using a luminometer to determine the transcriptional activation of the different PR.

SUMO assay. Detection of SUMOylated proteins is equally as difficult as the detection of ubiquitinated species. The use of NEM in the lysis buffer has proven to be a key step in the assay. SUMOylated PR is now detectable in the Lange laboratory in response to ligand (Fig. 9).

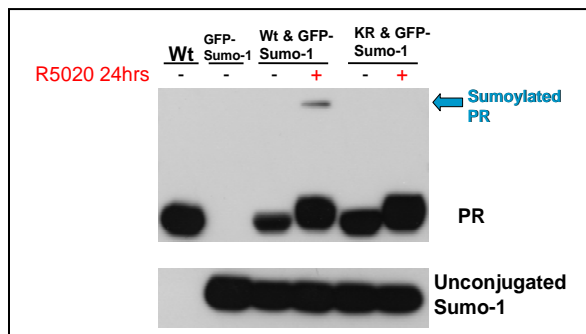
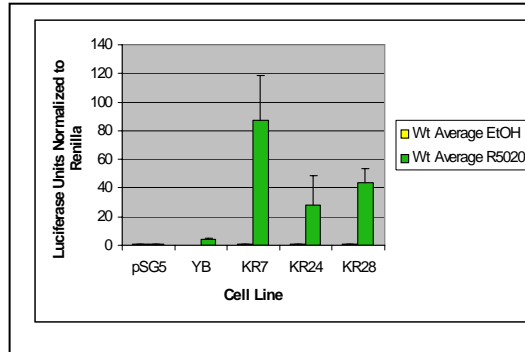


Fig. 9. Wt PR is SUMOylated in response to ligand whereas the K388R mutant PR is not. Cos cells were transiently transfected with wt PR or K388R PR and GFP-tagged SUMO, starved for one day and treated with or without R5020 for 24 hours. The cells were then harvested in lysis buffer containing 5M NEM and western blots were probed with antibodies against PR and SUMO-1.

Phosphorylation of PR on S294 negatively regulates SUMOylation. PR S294 is clearly an important regulatory site. To investigate potential mechanisms for phosphorylation mediated hypersensitivity of PR we are looking at the relationship between phosphorylation, ubiquitination and SUMOylation. Phosphorylation of PR increases ubiquitination, and it is possible that unphosphorylated receptors are modified in a way that decreases their activity. They may be SUMOylated. To probe this question we made use of our well-characterized S294A PR phospho-mutant. In a SUMO assay more PR-S294A is SUMOylated compared to wt PR (Fig. 10).

SUMOylation may be causing the transcriptional repression of the S294A mutant PR. To answer this question, a double mutant PR-B S294A/K388R was constructed and stably expressed it in T47DY cells (Fig. 11). In the presence of progestins, S294A PR-B is retained in the nucleus tightly associated with the nuclear matrix, is a poor transcription factor relative to wt PR-B and is resistant to ligand-dependent down-regulation. Mutation of K388R fully

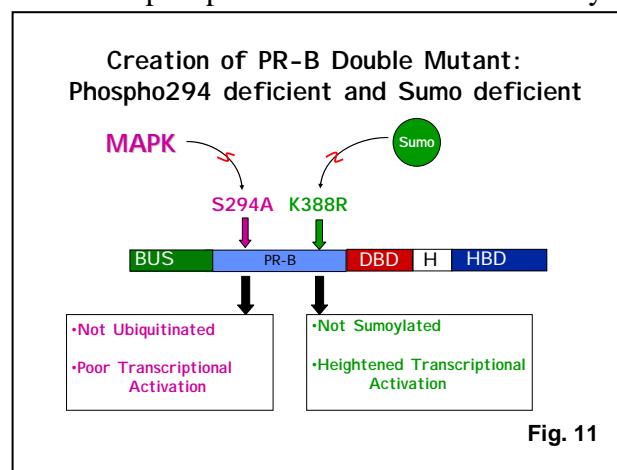


Fig. 11

rescued the S294A mutant phenotype; transcriptional activity was restored to high levels observed in the K388R mutant (Fig. 12A), and this receptor underwent ligand-dependent downregulation (Fig. 12B). These data indicate that without phosphorylation on S294 PR is SUMOylated in the presence of ligand, accounting for the phenotype of the S294A mutant.

Fig. 10. PR-S294A is SUMOylated. Cos cells were transiently cotransfected with wt PR, PR-K388R or PR-S294A and GFP-SUMO-1. Cells were starved, treated with or without R5020 for 24 hours and harvested in lysis buffer containing 5M NEM. Western blots were probed with antibodies against PR and SUMO-1.

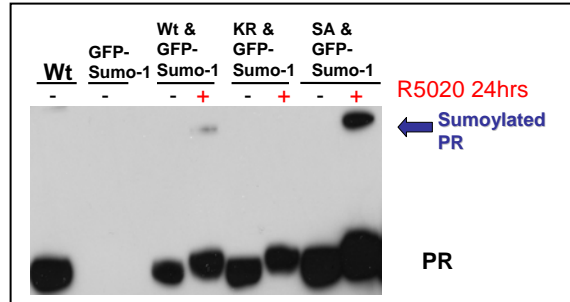
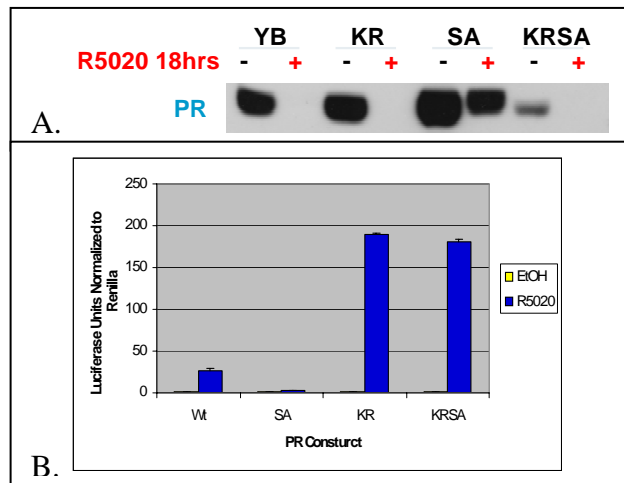


Fig. 12. SUMO mutant phenotype dominates the double mutant PR K388R/S294A. **A.** T47D-Y cells stably expressing wt PR-B (YB) and mutants were treated for 18 hours with or without R5020 and western blots were probed for PR. **B.** Luciferase Assays were performed on HeLa cells cotransfected with either wt PR-B, S294A PR-B, K388R PR-B or K388R/S294A PR-B, PRE-luciferase reporter constructs and Renilla control plasmid. Cells were treated for 24hrs with or without R5020 (10nM).



Ubiquitin and SUMO do not appear to compete for the same PR lysine. To explore the possibility that ubiquitin and SUMO are competing for conjugation to the same lysine in the PR sequence we looked at the ability of the K388R mutant to be degraded through the ubiquitin proteasome pathway. SUMO is conjugated to only one lysine within PR, K388; if mutation of this site does not abolish ubiquitination then other lysines in PR can be ubiquitinated. Direct competition for modification on lys388 is thus not occurring. T47DY cells expressing wt PR, the PR-K388R mutant or the PR-K388R/S294A mutant were treated with or without ligand and MG132, a proteasome inhibitor (Fig. 13). MG132 inhibited degradation of both wt and mutant PR in response to ligand, suggesting that the lysine at position 388 is not critical for ubiquitination and degradation by the proteasome pathway. PR-K388R/S294A appears to undergo proteasome dependent downregulation despite the lack of phosphorylation on S294, further implicating SUMOylation as a critical regulatory modification for the S294A mutant.

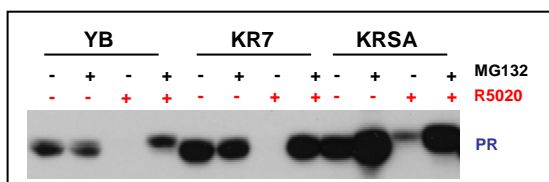


Fig. 13. MG132 inhibits degradation of K388R mutant PR. T47D-Y cells stably expressing wt, K388R or K388R/S294A PR were pretreated for 30 min with or without MG132 and treated for 18 hours with R5020. Western blots were probed for PR.

Key Research Accomplishments

- Generation of PR-B mutants to study PR-B Phosphorylation, ubiquitination and SUMOylation.
- Characterization of the mutants by PRE transcriptional reporter assays and by western blot.
- Development of ubiquitin and SUMO assays to characterize the post-translational modification of wt and mutant PR-Bs.
- Generation of T47D-Y cell lines stably expressing mutant PR-Bs.

Reportable Outcomes

Meetings and Abstracts

- Andrea R. Daniel and Carol A. Lange. Sumoylation of Progesterone Receptors (PR) is Negatively Regulated by Phosphorylation of PR Ser294. 88th Annual Meeting of The Endocrine Society, June 2006. *Poster Presentation.*
- Andrea R. Daniel, Carol A. Lange. Sumoylation of Progesterone Receptors (PR) is Negatively Regulated by Phosphorylation of PR Ser294. Third International Conference on Ubiquitin, Ubiquitin-like Proteins and Cancer, February 2006. *Oral Presentation.*
- Andrea R. Daniel, Ming Qiu, Carol A. Lange. Sumoylation of the Progesterone Receptor is Required for Membrane-Initiated PR Signaling and Mediates Negative Regulation of PR Transcriptional Activity. Second Great Lakes Nuclear Receptor Conference, October 2005. *Poster Presentation.*

Awards

- Women in Endocrinology Abstract Award (2006)
- University of Minnesota Department of Medicine's Research Day Poster Competition Award (2006)
- Second Great Lakes Nuclear Receptor Conference Abstract Award (2005)

Conclusion

We hypothesize phosphorylation of PR by growth factor pathways differentially regulates ubiquitination and SUMOylation of the receptor mediating changes in hormone responsiveness of breast cancer cells. We have shown that Phosphorylation on S294 PR-B is a positive regulator of ubiquitination and transcriptional activity of the receptor while a negative regulator of SUMOylation. SUMOylation of PR-B decreases its transcriptional activity. These studies provide a potential mechanism for the hypersensitivity of PR-B transcriptional activity in response to growth factor pathway

activation. Further investigation into the regulation of phosphorylation, ubiquitination and SUMOylation of PR-B will aid in the understanding of PR action in breast cancer.

Significance. Progestins are widely used in the clinic in HRT and birth control. Determining which PR actions mediate the positive therapeutic effects and the negative tumorigenic effects can lead to better therapies. With further investigation of the events which regulate PR we can identify the molecules and the mechanisms of action that cause the increased transcriptional activity of PR linked to its degradation. Furthermore, we may discover the pathways that are corrupted in breast cancers with altered hormone responsiveness, perhaps by creating rapidly turning over hypersensitive PR. This information will aid in the identification of new drug targets and/or optimization of existing therapies aimed at the inhibition of these molecules. The generation of ligands that antagonize certain actions and activate others could be useful, or inhibitors of modification pathways such as ubiquitination or SUMOylation may shift the balance of PR action toward positive effects.

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